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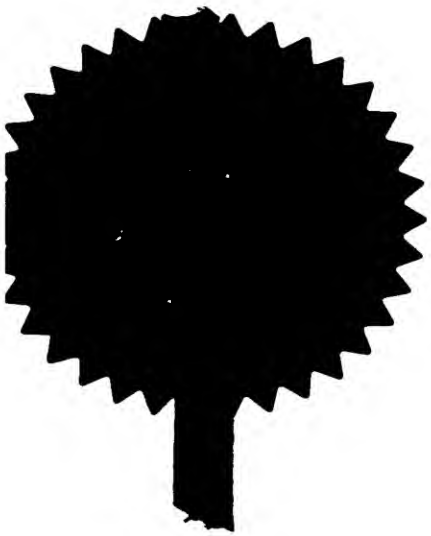

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	Patents ADP number *(if you know it)*	5319390001
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Cloning and Expression of a Novel Receptor

The present invention is concerned with cloning and expression of a novel receptor and, in particular, with a novel nucleic acid sequence encoding a 5-HT_4 splice variant designated herein as 5-$HT_{4(h)}$, an expression vector comprising said nucleic acid sequence, a host cell transformed or transfected with said vector, the 5-$HT_{4(h)}$ receptor protein expressed from said host cell and pharmaceutical compositions comprising said expressed protein or said nucleic acid or its complementary sequences.

The 5-HT_4 receptor is widely distributed in the body, in the periphery as well as in the central nervous system. In the periphery it is found in the gastrointestinal tract, for example in the esophagus (Moummi et al., 1992), the ileum (Buchheit and Buhl, 1991) and colon (Elswood et al., 1991). It is also present in the atrium (Kaumann.et al., 1990), the bladder (Candura et al., 1996) and the adrenal glands. In the rat brain, 5-HT_4 mRNA has been discovered by in situ hybridization in the olfactory tubercle, the striatum and the hippocampus (Vilaro et al., 1996). The wide distribution in different tissues of the 5-HT_4 receptor is parallelled by an also wide variety of 5-HT_4 variants caused by alternative splicing of exons. The splice variants described so far (Gerald et al., 1995; Claeysen et al., 1996; Van den Wyngaert et al., 1997; Claeysen et al., 1997; Blondel et al., 1997; Blondel et al., 1998) are all variations of the cytoplasmic C-terminus.

The predicted protein structures encoded by cDNA sequences already known reveal seven transmembrane domains for the complete ORFs. In addition to their structure and 5-HT_4 receptor coupled signal transduction events (increase in cAMP formation, opening of K+ channels), 5-HT_4 receptors have also been classified as G-protein coupled receptors (GPCRs).

The present inventors have identified a novel human 5-HT_4 splice variant (h), which leads to the insertion of 14 amino acids into the second extracellular loop of the receptor protein and expressed the isolated full length cDNA transiently in mammalian cells in order to compare its pharmacology with already known 5-HT_4 splice variants and its tissue distribution is analyzed by RT-PCR.

Therefore, according to a first aspect of the present invention, there is provided, an isolated nucleic acid encoding a human 5-$HT_{4(h)}$ receptor having the amino acid sequence illustrated in Figure 1b or encoding a functional equivalent, derivative or bioprecursor of said receptor. Advantageously, the isolated nucleic acid according to the invention may be used for expression in, for example, a host cell or the like using a suitable expression vector. Preferably, the nucleic acid may be a DNA molecule or a cDNA molecule. Preferably, the DNA molecule has the nucleic acid sequence as illustrated in Figure 1a.

According to a further aspect of the present invention, there is also provided an antisense

molecule comprising a nucleic acid sequence which is capable of hybridising to the nucleic acid sequence of the 5-$HT_{4(h)}$ receptor according to the invention under conditions of high stringency. Stringency of hybridisation as used herein refers to conditions under which polynucleic acids are stable. The stability of hybrids is reflected in the melting temperature (Tm) of the hybrids. Tm can be approximated by the formula:

$$81.5°C+16.6(\log_{10}[Na^+]+0.41\ (\%G\&C)-6001/l$$

wherein l is the length of the hybrids in nucleotides. Tm decreases approximately by 1-1.5°C with every 1% decrease in sequence homology.

The nucleic acid capable of hybridising to nucleic acid molecules according to the invention will generally be at least 70%, preferably at least 80 or 90% and more preferably at least 95% homologous to the nucleotide sequences according to the invention.

Advantageously, the antisense molecule may be used as a probe or as a medicament or in a pharmaceutical composition together with a pharmaceutically acceptable carrier, diluent or excipient.

According to a further aspect of the invention, there is provided a DNA expression vector comprising the DNA molecule according to the invention. This vector may advantageously be used to transform or transfect a host cell to achieve expression of the 5-$HT_{4(h)}$ receptor from said cell. Preferably, the DNA molecule

is included in a plasmid such as, for example, pcDNA3 for subsequent transformation or transfection of said host cell.

An expression vector according to the invention includes a vector having a nucleic acid according to the invention operably linked to regulatory sequences, such as promoter regions, that are capable of effecting expression of said DNA fragments. The term "operably linked" refers to a juxta position wherein the components described are in a relationship permitting them to function in their intended manner. Such vectors may be transformed into a suitable host cell to provide for expression of a polypeptide according to the invention. Thus, in a further aspect, the invention provides a process for preparing polypeptides according to the invention which comprises cultivating a host cell, transformed or transfected with an expression vector as described above under conditions to provide for expression by the vector of a coding sequence encoding the polypeptides, and recovering the expressed polypeptides.

The vectors may be, for example, plasmid, virus or phage vectors provided with an origin of replication, optionally a promoter for the expression of said nucleotide and optionally a regulator of the promoter. The vectors may contain one or more selectable markers, such as, for example, ampicillin resistance.

Regulatory elements required for expression include promoter sequences to bind RNA polymerase and

transcription initiation sequences for ribosome binding. For example, a bacterial expression vector may include a promoter such as the lac promoter and for transcription initiation in the Shine-Dalgarno sequence and the start codon AUG. Similarly, a eukaryotic expression vector may include a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors may be obtained commercially or assembled from the sequences described by methods well known in the art.

Nucleic acid molecules according to the invention may be inserted into the vectors described in an antisense orientation in order to provide for the production of antisense RNA. Antisense RNA or other antisense nucleic acids may be produced by synthetic means.

In accordance with the present invention, a defined nucleic acid includes not only the identical nucleic acid but also any amino base variations including, in particular, substitutions in bases which result in a synonymous codon (a different codon specifying the same amino acid residue) due to the degenerate code in conservative amino acid substitutions. The term "nucleic acid sequence" also includes the complementary sequence to any single stranded sequence given regarding base variations.

The present invention also advantageously provides nucleic acid sequences of at least approximately 10 contiguous nucleotides of a nucleic acid according to

the invention and preferably from 10 to 50 nucleotides. These sequences may, advantageously, be used as probes or primers to initiate replication, or the like. Such nucleic acid sequences may be produced according to techniques well known in the art, such as, by recombinant or synthetic means. They may also be used in diagnostic kits or the like for detecting the presence of a nucleic acid according to the invention. These tests generally comprise contacting the probe with the sample under hybridising conditions and detecting for the presence of any duplex or triplex formation between the probe and any nucleic acid in the sample.

According to the present invention these probes may be anchored to a solid support. Preferably, they are present on an array so that multiple probes can simultaneously hybridize to a single biological sample. The probes can be spotted onto the array or synthesised *in situ* on the array. (See Lockhart *et al.*, Nature Biotechnology, vol. 14, December 1996 "Expression monitoring by hybridisation into high density oligonucleotide arrays". A single array can contain more than 100, 500 or even 1,000 different probes in discrete locations.

The nucleic acid sequences, according to the invention may be produced using such recombinant or synthetic means, such as, for example, using PCR cloning mechanisms which generally involve making a pair of primers, which may be from approximately 10 to 50 nucleotides to a region of the gene which is desired to be cloned, bringing the primers into contact with

mRNA, cDNA, or genomic DNA from a human cell, performing a polymerase chain reaction under conditions which bring about amplification of the desired region, isolating the amplified region or fragment and recovering the amplified DNA. Generally, such techniques as defined herein are well known in the art, such as described in Sambrook *et al* (Molecular Cloning: a Laboratory Manual, 1989).

The nucleic acids or oligonucleotides according to the invention may carry a revealing label. Suitable labels include radioisotopes such as ^{32}P or ^{35}S, enzyme labels or other protein labels such as biotin or fluorescent markers. Such labels may be added to the nucleic acids or oligonucleotides of the invention and may be detected using known techniques *per se*.

The present invention also comprises within its scope proteins or polypeptides encoded by the nucleic acid molecules according to the invention or a functional equivalent, derivative or bioprecursor thereof. Preferably, the protein comprises the amino acid sequence illustrated in Figure 1B.

A "functional equivalent" as defined herein should be taken to mean a receptor that exhibits the same properties and functionality associated with the $5HT_{4(h)}$ receptor according to the invention. A "derivative" should be taken to mean a polypeptide or protein in which certain amino acids may have been altered or deleted or replaced and which polypeptide or protein retains biological activity of said $5HT_{4(h)}$ receptor and/or which can cross react with antibodies

raised using a receptor according to the invention as the challenging antigen.

Encompassed with the scope of the invention are hybrid and modified forms of the $5HT_{4(h)}$ receptor according to the invention including fusion proteins and fragments. The hybrid and modified forms include, for example, when certain amino acids have been subjected to some modification or replacement, such as for example, by point mutation and yet which results in a protein which possesses the same receptor specificity as the $5HT_{4(h)}$ of the invention.

The protein according to the invention should be taken to include all possible amino acid variants encoded by the nucleic acid molecule according to the invention including a polypeptide encoded by said molecule and having conservative amino acid changes. Proteins or polypeptides according to the invention further include variants of such sequences, including naturally occurring allelic variants which are substantially homologous to said proteins or polypeptides. In this context, substantial homology is regarded as a sequence which has at least 70%, and preferably 80 or 90% amino acid homology with the proteins or polypeptides encoded by the nucleic acid molecules according to the invention.

Further provided by the present invention is a transgenic cell, tissue or organism comprising a transgene capable of expressing a human $5\text{-}HT_{4(h)}$ receptor according to the invention, or expressing a functional equivalent, fragment, derivative or

bioprecursor of said receptor. The term "transgene capable of expression" as used herein means a suitable nucleic acid sequence which leads to the expression of a human 5-$HT_{4(h)}$ receptor having the same function and/or activity. The transgene may include, for example, genomic nucleic acid isolated from human cells or synthetic nucleic acid including cDNA integrated into the genome or in an extra chromosomal state. Preferably, the transgene comprises the nucleic acid sequence encoding the 5-$HT_{4(h)}$ receptor as described above, or a functional fragment of said nucleic acid. A functional fragment of said nucleic acid should be taken to mean a fragment of the gene comprising said nucleic acid, coding for the 5-$HT_{4(h)}$ receptor or a functional equivalent, derivative or bioprecursor of said receptor. For example, the gene may comprise deletions or mutations but may still encode a functional 5-$HT_{4(h)}$ receptor protein.

There is also provided by a further aspect of the present invention, a purified human 5-$HT_{4(h)}$ receptor expressed by a host cell or a transgenic cell tissue or organism as described herein or a functional equivalent, derivative or bioprecursor thereof.

The nucleic acid or protein according to the invention may be used as a medicament or in the preparation of a medicament for treating cancer or other diseases or conditions associated with expression of 5-$HT_{4(h)}$ receptor protein.

Advantageously, the nucleic acid molecule or the protein according to the invention may be provided in

a pharmaceutical composition together with a pharmacologically acceptable carrier, diluent or excipient therefor.

The present invention is further directed to inhibiting 5-$HT_{4(h)}$ *in vivo* by the use of antisense technology. Antisense technology can be used to control gene expression through triple-helix formation or antisense DNA or RNA, both of which methods are based on binding of a polynucleotide to DNA or RNA. For example, the 5′ coding portion of the mature protein sequence, which encodes for the protein of the present invention, is used to design an antisense RNA oligonucleotide of from 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription (triple-helix - see Lee *et al.* Nucl. Acids Res., 6:3073 (1979); Cooney *et al.*, Science, 241:456 (1988); and Dervan *et al.*, Science, 251: 1360 (1991), thereby preventing transcription and the production of 5-$HT_{4(h)}$. The antisense RNA oligonucleotide hybridises to the mRNA *in vivo* and blocks translation of an mRNA molecule into the 5-$HT_{4(h)}$ receptor.

A further aspect of the invention comprises the host cell itself transformed with the DNA expression vector described herein, which host cell preferably comprises a mammalian cell such as, for example, a COS-7 cell or a human cell such as a human embryonic kidney (HEK) 293 cell or the like.

Incorporation of cloned DNA into a suitable expression vector for subsequent transformation of the cell and

subsequent selection of the transformed cells is well known to those skilled in the art as provided in Sambrook *et al.*, (1989) "Molecular Cloning, A Laboratory Manual, Cold Spring Harbour Laboratory Press".

Antibodies to the human 5-$HT_{4(h)}$ receptor are also provided which may be used in a medicament or in a pharmaceutical composition.

Antibodies to the protein or polypeptide of the present invention may, advantageously, be prepared by techniques which are known in the art. For example, polyclonal antibodies may be prepared by inoculating a host animal, such as a mouse, with the polypeptide according to the invention or an epitope thereof and recovering immune serum. Monoclonal antibodies may be prepared according to known techniques such as described by Kohler R. and Milstein C., Nature (1975) 256, 495-497.

Antibodies according to the invention may also be used in a method of detecting for the presence of a receptor according to the invention, which method comprises reacting the antibody with a sample and identifying any protein bound to said antibody. A kit may also be provided for performing said method which comprises an antibody according to the invention and means for reacting the antibody with said sample.

Advantageously, the antibody according to the invention may also be used as a medicament or in the preparation of a medicament for treating diseases

associated with expression of 5-$HT_{4(h)}$. The invention also further provides a pharmaceutical composition comprising said antibody together with a pharmaceutically acceptable carrier, diluent or excipient therefor.

Proteins which interact with the polypeptide of the invention may be identified by investigating protein-protein interactions using the two-hybrid vector system first proposed by Chien *et al* (1991).

This technique is based on functional reconstitution *in vivo* of a transcription factor which activates a reporter gene. More particularly the technique comprises providing an appropriate host cell with a DNA construct comprising a reporter gene under the control of a promoter regulated by a transcription factor having a DNA binding domain and an activating domain, expressing in the host cell a first hybrid DNA sequence encoding a first fusion of a fragment or all of a nucleic acid sequence according to the invention and either said DNA binding domain or said activating domain of the transcription factor, expressing in the host at least one second hybrid DNA sequence, such as, a library or the like, encoding putative binding proteins to be investigated together with the DNA binding or activating domain of the transcription factor which is not incorporated in the first fusion; detecting any binding of the proteins to be investigated with a protein according to the invention by detecting for the presence of any reporter gene product in the host cell; optionally isolating second hybrid DNA sequences encoding the binding protein.

Proteins which bind to the 5-$HT_{4(h)}$ receptor can be identified using this technique. The proteins identified can also be used to identify compounds which acts as agonists/antagonists of these proteins. The structure of the receptor can also be used to design agonists or antagonists of the receptor. The present invention also comprises an agonist or antagonist of the human 5-$HT_{4(h)}$ receptor according to the invention which agonist or antagonist advantageously may also be used as a medicament or in a pharmaceutical composition together with a pharmaceutically acceptable carrier diluent or excipient therefor.

Further provided by the present invention is a method of determining whether a compound is an agonist or an antagonist of a 5-$HT_{4(h)}$ receptor protein, which method comprises contacting a host cell or transgenic cell tissue or organism according to the invention expressing said 5-$HT_{4(h)}$ receptor protein with said compound in the presence of a protein which binds to said receptor and monitoring induced cAMP formation in said cell. Preferably, the cell is a mammalian cell such as a COS-7 cell or the like or a human cell, such as a human embryonic kidney (HEK) 293 cell or the like. A further method of determining whether a compound is an agonist or an antagonist of 5-$HT_{4(h)}$ ligand protein is provided which method comprises contacting a cell or membrane preparation of said host cell or said transgenic cell according to the invention with said compound and establishing the binding affinity of said compound for said receptor. Any compounds identified may advantageously be used as

a medicament or in a pharmaceutical composition together with an appropriate diluent or excipient.

Further provided by the present invention is a kit for determining whether a compound is an agonist or an antagonist of a 5-$HT_{4(h)}$ receptor ligand, which kit comprises a cell according to the invention means for contacting said compound with said cell and means for measuring cAMP formation in said cell.

Disorders mediated by activation or expression of the $5HT_{4(h)}$ receptor may, advantageously, be treated by administering to an individual an amount of a compound identified as an agonist of the ligand binding $5HT_{4(h)}$ in sufficient concentration to reduce or prevent the symptoms of the disorder.

The present invention may be more clearly understood from the following exemplary embodiment with reference to the accompanying figures wherein;

Figure 1: a) is an alignment of nucleotide sequences of dog and human 5-$HT_{4(h)}$. The positions of primers used in this study are indicated by arrows and b) is an alignment of amino acid sequences of dog and human 5-$HT_{4(h)}$.

Figure 2: is an illustration of mRNA tissue distribution performed as described in Materials and Methods. The letters indicate the used primer combination in the PCR, A:FW AB1/REV B1 (5'part 5-HT_4

cDNA including h exon), B:FW AB1/REV AB2 (common part of all 5-HT_4 splice variants), C:FW AB2/REV SH1 (3'part 5-HT_{4a} cDNA), D:FW AB2/REV LO1 (3'part 5-HT_{4b} cDNA), E:FW B1/REV SH1 (3'part 5-HT_4 cDNA, combination of exon h and a), F:FWB1/REV LO1 (3'part 5-$HT_{4(h)}$).

Figure 3A: Saturation analysis of [^{3}H]GR113808 binding on membrane preparation from COS-7 cells transfected with the h 5-$HT_{4(h)}$. B. Saturation analysis of [^{3}H]5-HT binding on membrane preparation from COS-7 cells transfected with the h 5-$HT_{4(h)}$.

Figure 4: Inhibition of specific [^{3}H]GR113808 binding by 5-HT_4 agonist and antagonist. Membrane preparations from COS-7 cells transiently transfected with h 5-$HT_{4(h)}$ receptor were incubated with 0.25 nM [^{3}H]GR113808. Non-specific binding was determined by 10 mM SB204070. Results are percentages, 100% is defined by specific binding in the absence of competing compound. Results are the mean of three independent experiments from three different transfections. Calculated pIC50 values are given in Table 1.

Figure 5: Indirect estimation of AC stimulation by measuring cAMP formation in COS-7 cells transiently transfected with h 5-

$HT_{4(h)}$. Results represent the increase of cAMP after stimulation by agonist since basal level have been removed. Results are the mean of three independent experiments from three different transfections. Calculated pEC50 and % of 5-HT_{max} values are given in Table 2. The efficacy and potency of the different agonists to trigger the cellular response was estimated and compared for the three different variants. The mean of pEC50 and the percentage of stimulation, normalized for the maximum stimulation induced by 5-HT (% of 5-HT maximum) for the h5-$HT_{4(h)}$, h5-$HT_{4(a)}$ and h 5-$HT_{4(b)}$, are presented in Table 2. No difference in the pEC50 was noticed. The cAMP assay has been performed also for COS-7 cells transfected with the empty vector as a negative control. After stimulation with 10^{-6} M of each agonist, 5-HT, cisapride and prucalopride, no significant increase of the cAMP basal level was found.

Materials and Methods

Materials

AmpliTaq Gold, dNTPs, $MgCl_2$, and PCR bufferII were obtained from Perkin-Elmer Cetus (Foster City, CA, U.S.A.). T4 DNA ligase and restriction endonucleases were products of Boehringer (Mannheim, Germany). The Multiprime DNA labeling system and [^{3}H]GR 113808 with a specific activity of 3.07 TBq/mmol were obtained from Amersham (Little Chalfort, U.K.). The ExpressHybTM hybridization solution and the Smart cDNA synthesis kit were from Clontech (Palo Alto, CA, U.S.A.). [^{32}P] dCTP was purchased from NEN DuPont (Wilmington, DE, U.S.A.). Plasmid preparation kits and the Qiaquick PCR amplification kit were from Qiagen (Hilden, Germany). The PRISM Ready Reaction Dye Terminator Cycle Sequencing kits and the ABI 377 or 373A sequencing machines were from Applied Biosystems (Foster City, CA, U.S.A.). The Geneamp PCR System 9600 was from Perkin-Elmer (Norwalk, CT, U.S.A.). The mammalian expression vector pcDNA3 was obtained from Invitrogen (Carlsbad, CA, U.S.A.). Dulbecco's modified Eagle medium (DMEM) and foetal calf serum were from Life Technologies (Gaithersburg, MD, U.S.A.). The Bradford protein assay was performed with the reagent supplied from Bio-Rad (Nazareth Eke, Belgium), which also supplied the Zeta-Probe blotting membrane. The NEN flash plate assay was supplied by DuPont de Nemours (Brussels, Belgium). The liquid scintillation spectrometer and the scintillation fluid Ultima Gold MV were from Packard (Meriden, CT, U.S.A.). All compounds were dissolved and diluted in dimethyl

sulfoxide (DMSO; except the indoleamines, which were dissolved in water and protected from light throughout the experiment). The final DMSO concentration in the test did not exceed 0.5% (vol/vol). The GraphPad Prism program was from GraphPad Software, Inc. (San Diego, CA, U.S.A.).

General molecular biological methods

Unless otherwise indicated, all PCR reactions were performed in a total volume of 50 ml, containing 1 ml of cDNA and 1.25 U of AmpliTaq Gold in 1x PCR buffer II, 200 mM dNTPs, 400 nM primers, and 2.0 mM $MgCl_2$. PCR conditions were 10 min of denaturation at 95° C, followed by 35 cycles of 10 seconds at 95° C, 30 seconds at 53° C, and 2 min at 72° C, followed by a 10 min incubation at 72° C. DNA manipulations were done according to standard protocols (Maniatis et al., 1982). DNA sequencing was carried out with reagents from the PRISM Ready Reaction Dye Terminator Kit and run on a GeneAmp PCR System 9600 according to the specifications of the supplier.

BAC library screening

A human genomic DNA library in pBeloBAC11, Research Genetics (Huntsville, AL, U.S.A.) was screened by PCR using two primers, FW AB3 5'CTTCATGGTCAACAAGCCCTAC 3' and REV AB2 5'CCCGTTGTAACATCTGGATTTGVYGGGC3', specific for the 5-HT_4 cDNA. The position of the primers on the cDNA sequence is indicated in Fig.1. The PCRs were set up as described above in a total volume of 30 ml, 1 ml of the BAC pools supplied by Research Genetics was

used as substrate. BAC DNA was prepared using the Qiagen Maxi preparation kit (Hilden, Germany).

PCR amplification of the 5' and 3' ends of the human 5-$HT_{4(h)}$ and assembly to a full length coding region

Based on the nucleotide sequence of the 5-$HT_{4(h)}$ specific exon, derived from BAC clone 228K23 (Research Genetics), 2 primers were designed. Forward primer FW B1 (5'GAAAGGAGTCTAAACCAAGGCCT3') and reverse primer REV B2 (5'CGCATGAAAA TCCTGGCCCAGGCCTTGGTT3') hybridizing at positions indicated in Fig. 1A. Primer FW B1 was combined with reverse primer REV 3non (5'CAAGCAGCAGCTTAGGACCTG3') and reverse primer REV B2 was combined with forward primer FW ONstart (5'CCACTC ATGCTTATTTCCTGTAATG3'). PCR reactions were set up on cDNA prepared from human lower esophageal sphincter using Advantage Taq and initial denaturation for 1 min at 95° C. The resulting PCR products were cloned into EcoRV cut and dephosphorylated pcDNA3 (Invitrogen, Carlsbad, CA, U.S.A.). PCR products representing 5' and 3' part of the 5-$HT_{4(h)}$ were blunted by Klenow treatment and subsequently digested with the restriction enzyme StyI before ligation into pcDNA3.

mRNA tissue distribution analysis

Total RNA from the different tissues analysed was prepared by the CsCl method, cDNA was prepared thereof using the Smart cDNA library kit from Clontech (Palo Alto, CA, U.S.A.), 0.5 ml of the reaction product was used per PCR. The tissue distribution experiments were

done by PCRs, using 3 different forward primers and 4 different reverse primers. One forward primer, FW B1 is specific for cDNAs containing the 5-$HT_{4(h)}$ exon, the two others FW AB1 (5'GRAAYAAGATGACCCCTCTRCGYATC3') and FW AB2 (5'GCCCRNCARATCCAGATGTTACAACG3') will amplify all 5-HT_4 messages. Among the four reverse primers, one REV AB2 will amplify all 5-HT_4 messages, the other three reverse primers are specific for 5-$HT_{4(a)}$ (REV SH1, 5'GTATGGGCARYTTCTCS AGT TCCTGRTGWTG3'), 5-$HT_{4(b)}$ (REV LO1, 5'GAASTTGCTGNVRGGTGRCACYGACTCTC3') and 5-$HT_{4(h)}$ (REV B2). The position of the primers is indicated in figure 1. The PCR reactions were separated on an agarose gel and blotted on Zeta-Probe blotting membrane. A ^{32}P-labeled probe (Multiprobe DNA labeling system (Amersham)) corresponding to the human 5-$HT_{4(b)}$ cDNA (Van den Wyngaert et al., 1997) was hybridized to the PCR products on the membrane according to the instructions of the ExpressHybTM user manual (Clontech, Palo Alto, CA, U.S.A.).

Expression of the human 5-$HT_{4(h)}$ receptor in mammalian cells and pharmacological characterization

COS-7 cells were grown in DMEM supplemented with 10% fetal calf serum. A large scale plasmid preparation of 5-$HT_{4(h)}$/pcDNA3 was made using the Qiagen large scale plasmid preparation kit. Plasmid DNA was transfected into COS-7 cells as described in Van den Wyngaert et al. (1997). 48 hours after transfection the cells were harvested and used for membrane preparation or cyclic AMP formation assays as described (Van den Wyngaert et al., 1997).

Membrane preparation

The transfected COS-7 cells were cultured on 150 mm Petri dishes and washed twice with ice-cold phosphate-buffered saline. The cells were then scrapped from the plates with a cell scraper, suspended in 50 mM Tris-HCl buffer, pH 7.4, and harvested by centrifugation for 10 min at 16000 g. The pellet was resuspended in 5 mM Tris-HCl, pH 7.4, and homogenized with an Ultra Turax homogenizer; the resulting membranes were collected by centrifugation for 20 min at 25000g. Membranes were stored at -70° C in 50 mM Tris-HCl buffer , pH 7.4, at a protein concentration of 1 mg/ml. The Bradford protein assay was used for protein determination with bovine serum albumin as a standard.

Radioligand binding

Assay mixtures (0.5 ml) contained 50 μl of the tritiated ligand, (either the 5-HT_4 antagonist [^{3}H]GR113808, or the agonist [^{3}H]5-HT), 0.4 ml of membrane preparation (at 0.012 mg/ml of protein for [^{3}H]GR113808 binding or 0.1mg/ml for [^{3}H]5-HT), and 50 μl solvent for total binding, or 50 μl of 10 mM SB204070 to determine non specific binding. The [^{3}H]GR113808 assay buffer was 50 mM HEPES/NaOH pH 7.5. The [^{3}H]5-HT assay buffer was Tris-HCl pH 7.4 containing 10 mM $MgCl_2$, 1 mM pargyline (monoamine oxidase inhibitor) and 1 mM paroxetine (5-HT transport inhibitor). The mixture was incubated 1 hour at 25 °C. The incubation was terminated by rapid filtration over Whatman GF/B filters presoaked in 0.15% polyethylenimine and three washing steps with 3 ml of

50 mM HEPES/NaOH pH 7.5 for [^{3}H]GR113808 binding, presoaked and three washing steps with 3 ml Tris-HCl pH 7.4 for [^{3}H]5-HT binding. Ligand concentration isotherms were obtained using 8 concentrations of [^{3}H]GR113808 form 20 pM to 0.8 nM, and for [^{3}H]5-HT either 8 concentrations from 0.2 nM to 6 nM or from 0.2 nM to 40 nM were chosen. Competition binding experiments were performed with 0.25 nM of [^{3}H]GR113808 and otherwise at the same conditions as for [^{3}H]GR113808 saturation binding. Ligand concentration binding isotherms (rectangular hyperbola) and sigmoidal inhibition curves were calculated by nonlinear regression analysis according to algorithms described by Oestreicher and Pinto (1987). The maximal number of binding sites (B_{max}) and equilibrium dissociation constant (K_D) of the radioligand and the pIC_{50} (negative logarithm of the concentration that inhibits 50% of specific binding by the radioligand) values of competitors were derived from the curve fitting. Apparent inhibition constant (Ki) values were calculated according to the equation of Cheng and Prusoff (1973). Graphs were prepared using the GraphPad Prism program.

Measurement of cAMP formation

These experiments were done using the NEN adenylyl cyclase activation flashplate assay, according to the supplier. Cells were removed from the Petri dishes with 3 ml EDTA (0.04% w/v) and resuspended with phosphate buffered saline without Ca^{2+} and Mg^{2+}. The cells were centrifuged at 1500 g for 5 minutes and the supernatants were removed. The pellet was resuspend in

stimulation buffer and diluted to a concentration of 10^6 cells/ml, 50 µl thereof were added per well of the flashplate (50000 cells/well). Compounds were diluted in PBS containing 1 mM pargyline and 1 mM paroxetine, and 50 µl of the resulting mixture was added per well, followed by an incubation for 20 minutes at 37° C. The final concentration of DMSO (whenever needed to dissolve the compounds) did not exceed 0.5% (vol/vol) and was also included in the corresponding control samples. The experiment was stopped and a direct cAMP[^{125}I] detection assay was performed by adding 100 µl of detection mix per well. After incubation for 24h at room temperature, counting was done in a Topcount (Packard).

Results

Cloning of the human 5-$HT_{4(h)}$ splice variant

In the course of cloning the canine 5-HT_4 receptor cDNA by degenerate primer PCR based on our human 5-$HT_{4(b)}$ sequence (Van den Wyngaert et al., 1997) we found a variant (Fig. 1), similar to the partial porcine 5-$HT_{4(h)}$ cDNA originally designated 5-HT_4B (published by Ullmer et al., 1995). In order to amplify this sequence also from human cDNA, we applied primers based on the d 5-$HT_{4(h)}$ specific sequence to different human cDNAs. However we succeeded only in amplifying the 5' part of a putative human homologue and did not receive PCR products for the 3' part of the ORF. In order to investigate, whether there is an extensive sequence diversity between the human and canine version of this exon, respectively whether this

exon exists at all in human, we screened a human genomic DNA BAC library for a clone containing the human 5-HT_4 gene. One positive clone, 228K23 (Research Genetics) was identified. DNA prepared from this clone was sequenced using primer FW B2 (5'AACCAAGGCCTGGGCCAGGATTTTC ATGGG3'), complementary to a part of the 5-$HT_{4(h)}$ exon. The resulting sequence stretched into the adjacent intron sequence, design of a reverse primer complementary to that intron sequence allowed complete sequence determination of the human 5-$HT_{4(h)}$ exon sequence. Based on this information, h-variant specific forward and reverse primers were designed and combined with primer FW ONstart and reverse primers specific for the a and b splice variant. However for the latter, only the b variant specific primer REV 3non produced a PCR product of expected size in combination with FW B1. The two PCR products were fused by using the unique StyI restriction enzyme site in the nucleotide sequence of the 5-$HT_{4(h)}$ specific exon to build a full length reading frame.

Tissue distribution of the 5-$HT_{(4h)}$ mRNA

In an initial experiment to explore the specific function of the 5-$HT_{4(h)}$ in human physiology, we performed a tissue distribution study. The primers were chosen in order to obtain PCR products from parts of the 5-HT_4 cDNA that are common to all different variants and also to obtain bands that are specific for the a, b or h exon (Fig.2). The only tissue from which detectable levels of a PCR product corresponding to the 5-$HT_{4(h)}$ variant, could be produced, was the

lower esophageal sphincter (LES). For a number of the examined tissues, we found PCR products corresponding to the presence of h exon mRNA, but not in combination with the a or b specific C-terminal exon, in these cases, the h specific exon may be fused to another C-terminal exon of the 5-HT_4 gene. All other examined tissues showed bands corresponding to either the a or b splice variant, but not to the h variant.

Transient expression of the 5-$HT_{4(h)}$ variant in mammalian cells and pharmacological characterization

In order to compare the pharmacological properties of the 5-$HT_{4(h)}$ variant with those of the previously cloned 5-$HT_{4(a)}$ and b variant, the three corresponding pcDNA3 expression constructs were transiently transfected into COS cells. The receptors were investigated by radioligand binding assays on membrane preparations. Saturation analysis experiments were performed with the agonist [^{3}H]5-HT as well as with the antagonist [^{3}H]GR113808. Antagonist and agonist saturation binding of the h variant from 3 independent transfections resulted in a straight line in Scatchard analysis, revealing a single high-affinity binding site. The ligand concentration isotherms of the h 5-$HT_{4(h)}$ revealed a K_D of 0.24 (+/-0.17) nM using the antagonistic ligand and 1.65 (+/-0.55) nM based on the agonistic ligand (Fig. 3A and B).

These values were not significantly different from the two other variants we investigated as reference, the K_D of the variants a and b were respectively 0.14 (+/-0.06) and 0.19 (+/-0.07) nM for [^{3}H]GR113808

saturation binding and 3.7 (+/-0.6) and 4.6 (+/-1.9) nM for [^{3}H]5-HT saturation binding. The h 5-$HT_{4(h)}$ receptor displayed a *B*max of 605 (+/- 174) fmol/mg of protein with [^{3}H]GR113808, and 61 (+/-12) fmol/mg of protein with [^{3}H]5-HT. Scatchard analysis from [^{3}H]5-HT binding suggested two affinity sites for the two 5-HT_4 receptor variants a and b. For each of the 3 independent transfections, COS-7 cells transfected with the vector alone showed no specific binding using the radioligand [^{3}H]GR113808. Saturation binding experiments with [^{3}H]GR113808 and [^{3}H]5-HT were performed in parallel for each of the 3 independent transfections. The resulting ratio of the *B*max found using the antagonistic radioligand versus the Bmax based on the agonistic radioligand reveals the proportion of the coupled receptors among the total number of receptors. We found this proportion to be 9.8 (+/-1.05) for the h5-$HT_{4(h)}$. The same result was obtained for the *B*max values found for the high affinity sites of the 2 reference 5-HT receptor variants a and b. However using the Bmax values obtained with the low affinity site, yielded a ratio of coupled receptors to total receptors that was 3 times lower. This result suggests a difference in the G-protein coupling of the h 5-$HT_{4(h)}$ variant, compared to the h 5-$HT_{4(a)}$ and h 5-$HT_{4(b)}$ variant. The pharmacological binding profile of the h5-$HT_{4(h)}$ was studied by competition binding assays using six different agonists and two antagonists in combination with the radioligand [^{3}H]GR113808 at a concentration of 0.25 nM (Fig. 4) on COS-7 membranes. Results are the mean of three independent transfections.

The 5-HT_4 receptor is suggested to be involved in a number of different physiological processes, which makes it an important pharmacological target. 5-HT_4 receptor activation influences gastrointestinal motility (Meulemans and Schuurkes, 1992), bladder function (Candura et al., 1996), exerts chronotropic and inotropic effects at the heart (Kaumann et al., 1990) and centrally enhances striatal dopamine release (Bonhomme et al., 1995) as well as associative memory in rats (Marchetti-Gauthier et al., 1997). This variety of physiological effects is paralleled by a variety of splice variants which have been discovered in the course of the last two years (Gerald et al., 1995; Claeysen et al., 1996; Van den Wyngaert et al., 1997; Claeysen et al., 1997; Blondel et al., 1997; Blondel et al., 1998). For these splice variants up to now no well documented specific biochemical or physiological properties described. However for splice variants of other GPCRs more information is available. For the C-terminal splice variants of the prostaglandin EP_3 receptor coupling to different G-proteins and signal transduction systems has been shown (Namba et al., 1993). C-terminal splice variants of the mouse somatostatin receptor differ in the efficiency of adenylate cyclase inhibition and receptor desensitization (Vanetti et al., 1993). Differential splicing at the third intracytoplasmic loop of the PACAP receptor leads to coupling to different G- proteins (Spengler et al., 1993), the same was found for C-terminal splice variants of mGluR1 (Pin et al. 1992). Pickering et al. (1993) showed for the same variants in addition differential intracellular distribution. For some of these GPCR

splice variants also differential tissue distribution has been shown (Spengler et al., 1993), also this is not the case for others (Pin et al., 1992). The up to now published 5-HT_4 splice variants all vary in their C-terminus, which suggests in analogy to the results obtained from other GPCRs that they may differ from each other in respect to G-protein usage, desensitization and/or subcellular localization. The 5-$HT_{4(h)}$ variant described in this study has an extra insertion of 14 amino acids in the second extracellular loop, to our knowledge there are no other descriptions of such a variation produced by alternative splicing. Surprisingly this modification led to the loss of the low affinity agonist binding site that was found for the a and b splice variant. Although the ratio of coupled to uncoupled receptor is still the same for the high affinity agonist binding site across all three known variants, it is likely that the insertion of 14 amino acids into the second extracellular loop leads to a change of receptor topology that is reflected in G-protein binding. This change in receptor topology is also suggested by the agonistic effect that GR113808 exerts at this variant, which acts as an antagonist at all other 5-HT_4 variants. The availability of a variety of 5-HT_4 splice variants offers the opportunity for medicinal chemistry to pursue a higher degree of specificity for drug development. Given that the standard 5-HT4 receptor antagonist GR 113 808 showed agonistic activity on the 5-HT4(h) receptor variant and that 5-HT4 receptor antagonists are under investigation as compounds for the treatment of irritable bowel syndrome (IBS), testing of any given ligand on the 5-

HT4(h) variant is essential before classifying it as an 5-HT4 receptor antagonist. Based on the specific tissue distribution of the 5-$HT_{4(h)}$ to the LES, compounds showing specificity towards that 5-HT_4 splice variant may have therapeutic value for the treatment of heartburn, reflux, irritable bowel syndrome, esophagitis, Barrett's esophagus, esophageal cancer, achalasia, esophageal stenosis, esophagel spasms, esophageal hiatal hernia or other esophageal motility disorders. Furthermore these compounds may be of value in the treatment of airway disorders possibly connected with oesophageal irritation, such as asthma, bronchospasms, aspiration and its consequences (bronchitis, (broncho)pneumonia, bronchiectasia).

Compounds acting on 5-HT_4 receptor splice variants found in the lower oesophageal sphincter, may additionally be useful in treating or alleviating the symptoms of diseases of the lower oesophageal sphincter or other conditions such as achalasia; oesophageal stenosis (due to systemic sclerosis, tumours, burns,...) or compression, oesophageal spasms or other oesophageal motility disorders, irritable bowel syndrome, asthma, bronchospasms and other airway disorders possibly connected with oesophageal irritation aspiration and its consequence (bronchitis, (broncho)pneumonia, bronchiectasia, ...); (hiatus) hernia; denervation of the oesophagus (e.g. after certain types of trauma or surgery), disturbances in oesophageal innervation; pregnancy (not a disease or even a condition that as such could be treated with 5HT4-receptor compounds, but one in which -for various reasons-oesophageal reflux and its consequences are

more common); emesis; postoperative ileus; diabetic gastroparesis.

Abbreviations used: AC, adenylyl cyclase; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide; 5-HT, 5-hydroxytryptamine, serotonin; Ki, inhibition constant; LES, lower esophagael sphincter; ORF, open reading frame.

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Table 1 Comparison of potency of compounds to compete with 0.25 nM [^{3}H]GR113808 binding in membranes from COS-7 cells transiently transfected with respectively human 5-$HT_{4(h)}$, 5-$HT_{4(a)}$ or 5-$HT_{4(b)}$ receptors.

	Human 5-HT4 h receptor in Cos-7 cells		Human 5-HT4 a receptor in Cos-7 cells		Human 5-HT4 b receptor in Cos-7 cells	
Compounds	pIC50 +/- SD (n)	pKi	pIC50 +/- SD (n)	pKi	pIC50 +/-SD (n)	pKi
Agonist						
5HT	6.09 +/- 0.05 (3)	6.43	6.80 +/- 0.37 (3)	6.89	6.47 +/- 0.18 (3)	6.63
Prucalopride	6.74 +/- 0.24 (3)	8.03	7.20 +/- 0.40 (4)	7.21	7.04 +/- 0.25 (4)	7.16
Cisapride	6.78 +/- 0.32 (3)	7.05	7.22 +/- 0.47 (4)	7.23	6.92 +/- 0.33 (4)	6.99
Mosapride	6.55 +/- 0.24 (3)	6.85	6.48 +/- 0.19 (3)	6.71	6.04 +/- 0.27 (3)	6.18
SDZ-HTF919	7.45 +/- 0.52 (3)	7.59	8.08 +/- 0.66 (4)	7.86	7.89 +/- 0.20 (4)	8.09
5-MeOT	5.82 +/- 0.27 (3)	6.1	6.09 +/- 0.53 (4)	6.08	6.11 +/- 0.52 (4)	6.13
Antagonist						
SB204070	10.01 +/- 0.17 (3)	10.38	10.07 +/- 0.32 (4)	10.1	9.98 +/- 0.16 (4)	10.04
GR113808	9.05 +/- 0.31 (3)	9.29		5	9.39 +/- 0.13 (4)	9.57
			9.45 +/- 0.35 (4)	9.54		

Table 2 Comparison of potency and affinity of agonist compounds to stimulate cAMP formation in COS-7 cells transiently transfected with respectively human 5-$HT_{4(h)}$, 5-$HT_{4(a)}$ or 5-$HT_{4(b)}$ receptors.

	% of 5HT max (+/-SD)			pEC50 (+/1 SD)		
5-HT4 variants	a	b	h	a	b	h
Compounds	n=5	n=8	n=3	n=5	n=8	n=3
5HT	100	100	100	8.24	8.31	7.9
	-	-	-	(+/-0.156)	(+/-0.147)	(+/-0.26)
Prucalopride	118	117.36	95	8.24	8.49	8.36
	(+/-7.2)	(+/-3.4)	(+/-11.6)	(+/-0.105)	(+/-0.049)	(+/-0.62)
Cisapride	98.17	113.62	123.53	7.82	7.9	7.99
	(+/-7.5)	(+/-4.36)	(+/-20.85)	(+/-0.26)	(+/-0.113)	(+/-0.62)
GR113808	0	0	54.6	-	-	7.95
			(+/-5.8)			(+/-0.38)

Claims

1. A nucleic acid molecule encoding a human 5-$HT_{4(h)}$ receptor having the amino acid sequence illustrated in Figure 1b or encoding a functional equivalent, derivative or bioprecursor of said receptor.

2. A nucleic acid molecule according to claim 1 which is a DNA molecule.

3. A nucleic acid molecule according to claim 2, wherein said DNA molecule is a cDNA molecule.

4. A nucleic acid molecule according to claim 2 or 3 comprising the sequence as illustrated in Figure 1a.

5. A human 5-$HT_{4(h)}$ receptor encoded by the nucleic acid molecule according to any of claims 1 to 4.

6. A DNA expression vector comprising a nucleic acid molecule according to any of claims 2 to 4.

7. A host cell transformed or transfected with the vector of claim 6.

8. A host cell according to claim 7, which cell is a mammalian cell.

9. A host cell according to claim 8, which mammalian cell is a COS-7 cell.

10. A transgenic cell, tissue or organism comprising a transgene capable of expressing a human 5-$HT_{4(h)}$ receptor protein having the amino acid sequence of Figure 1b or an amino acid sequence of a functional equivalent, derivative or bioprecursor of said receptor.

11. A human 5-$HT_{4(h)}$ receptor protein or a functional equivalent, derivative or bioprecursor thereof, expressed by the cell according to any of claims 7 to 9 or the cell tissue or organism according to claim 10.

12. A HEK 293 or COS-7 5-$HT_{4(h)}$ cell line transfected with the expression vector of claim 6.

13. An antisense molecule comprising a nucleic acid molecule which is capable of hybridising to the nucleic acid of any of claims 1 to 4 under conditions of high stringency.

14. A pharmaceutical composition comprising a molecule according to claim 13 together with a pharmaceutically acceptable carrier, diluent or excipient therefor.

15. An antisense molecule according to claim 13 for use as a medicament.

16. A purified human 5-$HT_{4(h)}$ receptor protein comprising the amino acid sequence as illustrated in Figure 1b or the amino acid sequence of a functional equivalent, derivative, fragment or

bioprecursor of said sequence.

17. An antagonist or an agonist of a ligand of the human 5-$HT_{4(h)}$ receptor protein according to any of claims 11 or 16.

18. A pharmaceutical composition comprising an antagonist or an agonist according to claim 17 together with a pharmaceutically acceptable carrier, diluent or excipient therefor.

19. A method of determining whether a compound is an agonist or an antagonist of a human 5-$HT_{4(h)}$ ligand protein, which method comprises contacting a cell according to any of claims 7 to 10 expressing said receptor protein with said compound in the presence of said ligand and monitoring cAMP formation in said cell.

20. A method according to claim 19 wherein said cell is a human cell.

21. A method of determining whether a compound binds to a human 5-$HT_{4(h)}$ receptor which method comprises contacting a cell, according to any of claims 7 to 10 or a membrane preparation comprising said receptor, with said compound and establishing the binding affinity of said compound for said receptor.

22. A compound identified as an agonist or antagonist according to the method of claim 20 or 21.

23. A compound according to claim 22 for use as a medicament.

24. Use of a compound identified according to claim 22 or an antisense molecule according to claim 15 in the manufacture of a medicament for the treatment of any of heartburn, reflux, esophagitis, Barrett's esophagus, esophageal cancer, achalasia, esophageal stenosis, esophagel spasms, esophageal hiatal hernia or other esophageal motility disorders, oesophageal irritation, such as asthma, bronchospasms, aspiration and its consequences (bronchitis, (broncho)pneumonia, bronchiectasia) and other diseases of the lower oesophageal sphincter, or achalasia; oesophageal stenosis (due to systemic sclerosis, tumours, burns, or the like) or compression, oesophageal spasms or other oesophageal motility disorders, asthma, irritable bowel syndrome, bronchospasms and other airway disorders possibly connected with oesophageal irritation aspiration and its consequence (bronchitis, (broncho)pneumonia, bronchiectasia); (hiatus) hernia; denervation of the oesophagus (e.g. after certain types of trauma or surgery), disturbances in oesophageal innervation.

25. A pharmaceutical composition comprising a compound according to claim 22 together with a pharmaceutically acceptable carrier diluent or excipient therefor.

26. An antibody specific for a human 5-$HT_{4(h)}$ receptor

according to claim 5 or 16.

27. A kit for determining whether a compound is an agonist or an antagonist of a 5-$HT_{4(h)}$ ligand, which kit comprises a cell according to any of claims 7 to 10, means for contacting said compound and said ligand with said cell and means for measuring cAMP formation is said cell.

28. A kit according to claim 27 wherein said cell is a COS-7 cell.

29. A pharmaceutical composition incorporating the nucleic acid sequence according to claims 1 to 4, or the antibody according to claim 26, together with a pharmaceutically acceptable carrier, diluent or excipient therefor.

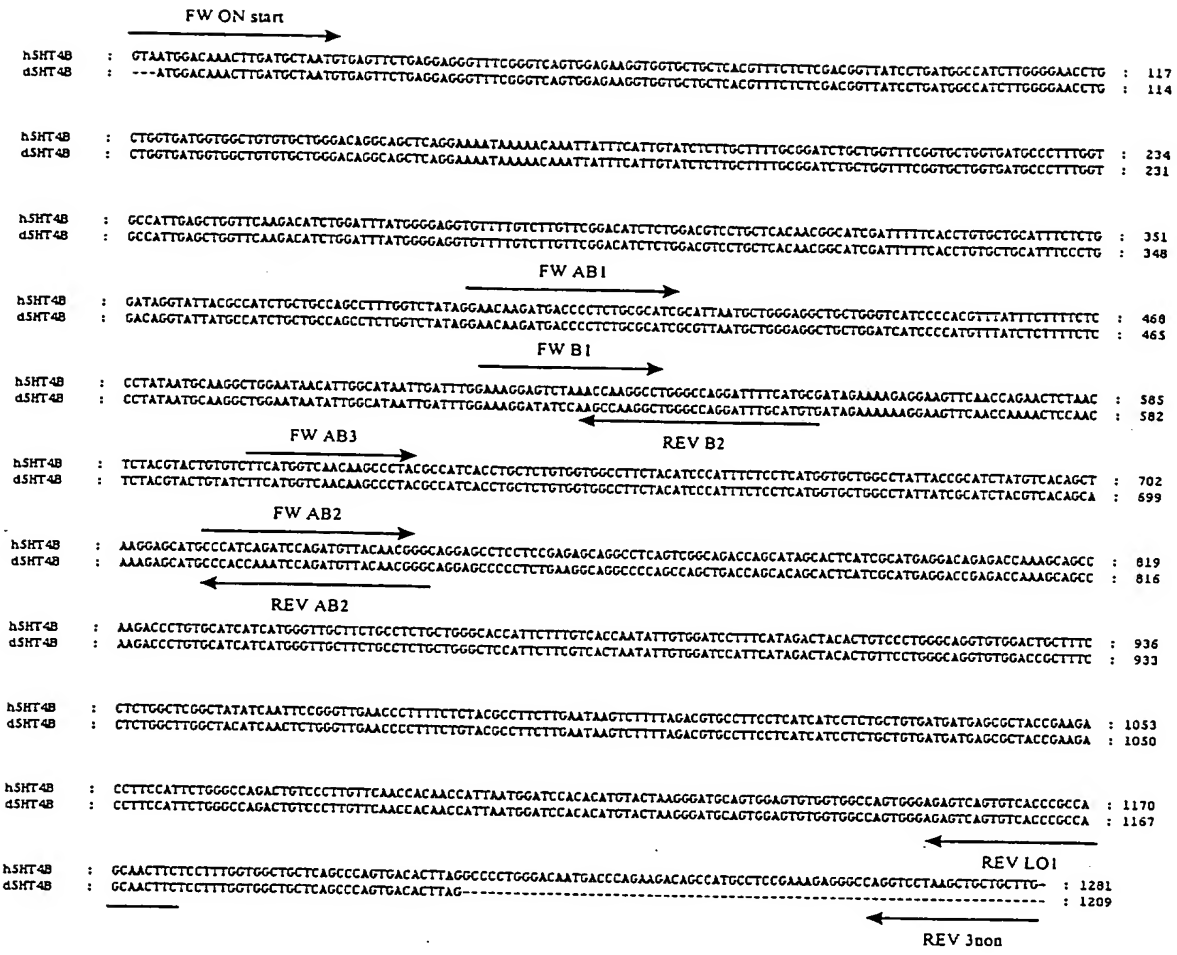
FW ON start
h5HT4B : GTAATGGACAAACTTGATGCTAATGTGAGTTCTGAGGAGGGTTTCGGGTCAGTGGAGAAGGTGGTGCTGCTCACGTTTCTCTCGACGGTTATCCTGATGGCCATCTTGGGGAACCTG : 117
d5HT4B : ---ATGGACAAACTTGATGCTAATGTGAGTTCTGAGGAGGGTTTCGGGTCAGTGGAGAAGGTGGTGCTGCTCACGTTTCTCTCGACGGTTATCCTGATGGCCATCTTGGGGAACCTG : 114
h5HT4B : CTGGTGATGGTGGCTGTGTGCTGGGACAGGCAGCTCAGGAAAATAAAAACAAATTATTTCATTGTATCTCTTGCTTTTGCGGATCTGCTGGTTTCGGTGCTGGTGATGCCCTTTGGT : 234
d5HT4B : CTGGTGATGGTGGCTGTGTGCTGGGACAGGCAGCTCAGGAAAATAAAAACAAATTATTTCATTGTATCTCTTGCTTTTGCGGATCTGCTGGTTTCGGTGCTGGTGATGCCCTTTGGT : 231
h5HT4B : GCCATTGAGCTGGTTCAAGACATCTGGATTTATGGGGAGGTGTTTTGTCTTGTTCGGACATCTCTGGACGTCCTGCTCACAACGGCATCGATTTTTCACCTGTGCTGCATTTCTCTG : 351
d5HT4B : GCCATTGAGCTGGTTCAAGACATCTGGATTTATGGGGAGGTGTTTTGTCTTGTTCGGACATCTCTGGACGTCCTGCTCACAACGGCATCGATTTTTCACCTGTGCTGCATTTCCCTG : 348
FW AB1
h5HT4B : GATAGGTATTACGCCATCTGCTGCCAGCCTTTGGTCTATAGGAACAAGATGACCCCTCTGCGCATCGCATTAATGCTGGGAGGCTGCTGGGTCATCCCCACGTTTATTTCTTTTCTC : 468
d5HT4B : GACAGGTATTATGCCATCTGCTGCCAGCCTCTGGTCTATAGGAACAAGATGACCCCTCTGCGCATCGCGTTAATGCTGGGAGGCTGCTGGATCATCCCCATGTTTATCTCTTTTCTC : 465
FW B1
h5HT4B : CCTATAATGCAAGGCTGGAATAACATTGGCATAATTGATTTGGAAAGGAGTCTAAACCAAGGCCTGGGCCAGGATTTTCATGCGATAGAAAAGAGGAAGTTCAACCAGAACTCTAAC : 585
d5HT4B : CCTATAATGCAAGGCTGGAATAATATTGGCATAATTGATTTGGAAAGGATATCCAAGCCAAGGCTGGGCCAGGATTTGCATGTGATAGAAAAAAGGAAGTTCAACCAAAACTCCAAC : 582
FW AB3
REV B2
h5HT4B : TCTACGTACTGTGTCTTCATGGTCAACAAGCCCTACGCCATCACCTGCTCTGTGGTGGCCTTCTACATCCCATTTCTCCTCATGGTGCTGGCCTATTACCGCATCTATGTCACAGCT : 702
d5HT4B : TCTACGTACTGTATCTTCATGGTCAACAAGCCCTACGCCATCACCTGCTCTGTGGTGGCCTTCTACATCCCATTTCTCCTCATGGTGCTGGCCTATTATCGCATCTACGTCACAGCA : 699
FW AB2
h5HT4B : AAGGAGCATGCCCATCAGATCCAGATGTTACAACGGGCAGGAGCCTCCTCCGAGAGCAGGCCTCAGTCGGCAGACCAGCATAGCACTCATCGCATGAGGACAGAGACCAAAGCAGCC : 819
d5HT4B : AAAGAGCATGCCCACCAAATCCAGATGTTACAACGGGCAGGAGCCCCCTCTGAAGGCAGGCCCCAGCCAGCTGACCAGCACAGCACTCATCGCATGAGGACCGAGACCAAAGCAGCC : 816
REV AB2
h5HT4B : AAGACCCTGTGCATCATCATGGGTTGCTTCTGCCTCTGCTGGGCACCATTCTTTGTCACCAATATTGTGGATCCTTTCATAGACTACACTGTCCCTGGGCAGGTGTGGACTGCTTTC : 936
d5HT4B : AAGACCCTGTGCATCATCATGGGTTGCTTCTGCCTCTGCTGGGCTCCATTCTTTGTCACTAATATTGTGGATCCATTCATAGACTACACTGTTCCTGGGCAGGTGTGGACCGCTTTC : 933
h5HT4B : CTCTGGCTCGGCTATATCAATTCCGGGTTGAACCCTTTTCTCTACGCCTTCTTGAATAAGTCTTTTAGACGTGCCTTCCTCATCATCCTCTGCTGTGATGATGAGCGCTACCGAAGA : 1053
d5HT4B : CTCTGGCTTGGCTACATCAACTCTGGGTTGAACCCCTTTCTGTACGCCTTCTTGAATAAGTCTTTTAGACGTGCCTTCCTCATCATCCTCTGCTGTGATGATGAGCGCTACCGAAGA : 1050
h5HT4B : CCTTCCATTCTGGGCCAGACTGTCCCTTGTTCAACCACAACCATTAATGGATCCACACATGTACTAAGGGATGCAGTGGAGTGTGGTGGCCAGTGGGAGAGTCAGTGTCACCCGCCA : 1170
d5HT4B : CCTTCCATTCTGGGCCAGACTGTCCCTTGTTCAACCACAACCATTAATGGATCCACACATGTACTAAGGGATGCAGTGGAGTGTGGTGGCCAGTGGGAGAGTCAGTGTCACCCGCCA : 1167
REV LO1
h5HT4B : GCAACTTCTCCTTTGGTGGCTGCTCAGCCCAGTGACACTTAGGCCCCTGGGACAATGACCCAGAAGACAGCCATGCCTCCGAAAGAGGGCCAGGTCCTAAGCTGCTGCTTG- : 1281
d5HT4B : GCAACTTCTCCTTTGGTGGCTGCTCAGCCCAGTGACACTTAG---------- : 1209
REV 3non

Fig 1a

Fig. 1B

```
h5HT4B  :  VMDKLDANVSSEEGFGSVEKVVLLTFLSTVILMAILGNLLVMVAVCWDRQLRKIKTNYFIVSLAFADLLVSVLVMPF  :   77
d5HT4B  :  -MDKLDANVSSEEGFGSVEKVVLLTFLSTVILMAILGNLLVMVAVCWDRQLRKIKTNYFIVSLAFADLLVSVLVMPF  :   76

h5HT4B  :  GAIELVQDIWIYGEVFCLVRTSLDVLLTTASIFHLCCISLDRYYAICCQPLVYRNKMTPLRIALMLGGCWVIPTFIS  :  154
d5HT4B  :  GAIELVQDIWIYGEVFCLVRTSLDVLLTTASIFHLCCISLDRYYAICCQPLVYRNKMTPLRIALMLGGCWIIPMFIS  :  153

h5HT4B  :  FLPIMQGWNNIGIIDLERSLNQGLGQDFHAIEKRKFNQNSNSTYCVFMVNKPYAITCSVVAFYIPFLLMVLAYYRIY  :  231
d5HT4B  :  FLPIMQGWNNIGIIDLERISKPRLGQDLHVIEKRKFNQNSNSTYCIFMVNKPYAITCSVVAFYIPFLLMVLAYYRIY  :  230

h5HT4B  :  VTAKEHAHQIQMLQRAGASSESRPQSADQHSTHRMRTETKAAKTLCIIMGCFCLCWAPFFVTNIVDPFIDYTVPGQV  :  308
d5HT4B  :  VTAKEHAHQIQMLQRAGAPSEGRPQPADQHSTHRMRTETKAAKTLCIIMGCFCLCWAPFFVTNIVDPFIDYTVPGQV  :  307

h5HT4B  :  WTAFLWLGYINSGLNPFLYAFLNKSFRRAFLIILCCDDERYRRPSILGQTVPCSTTTINGSTHVLRDAVECGGQWES  :  385
d5HT4B  :  WTAFLWLGYINSGLNPFLYAFLNKSFRRAFLIILCCDDERYRRPSILGQTVPCSTTTINGSTHVLRDAVECGGQWES  :  384

h5HT4B  :  QCHPPATSPLVAAQPSDTAPGTMTQKTAMPPKEGQVLSCCL  :  426
d5HT4B  :  QCHPPATSPLVAAQPSDT----------------------  :  402
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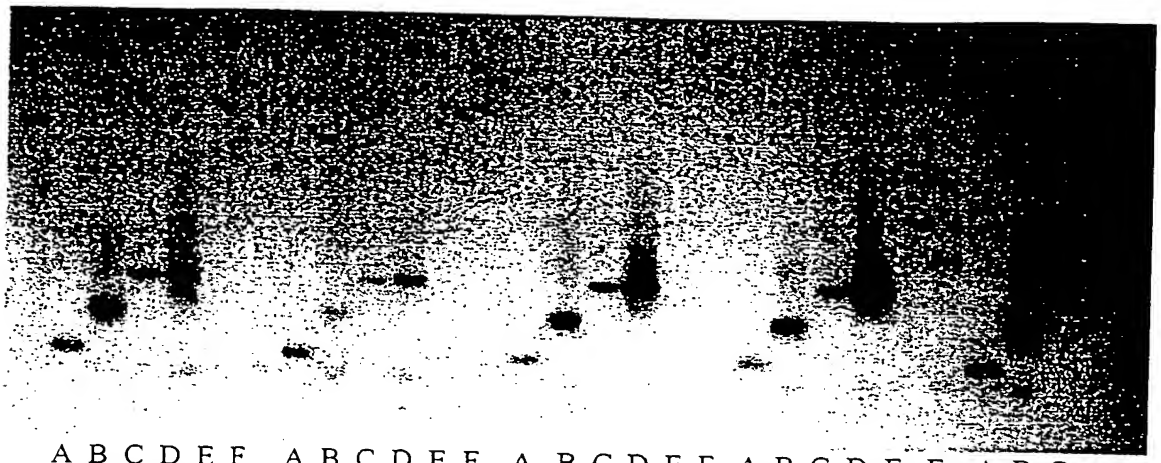
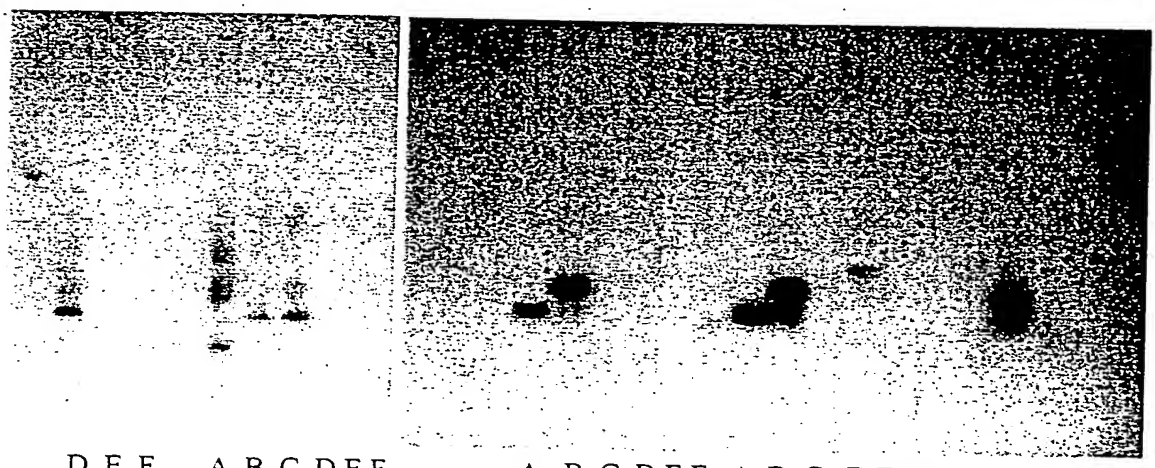


A B C D E F A B C D E F A B C D E F A B C D E F A B C
stomach esophagus adrenal glands LES small intestine



D E F A B C D E F A B C DE F A B C DE F A B C DE F
small intestine large intestine hippocampus cerebellum striatum

Fig 2

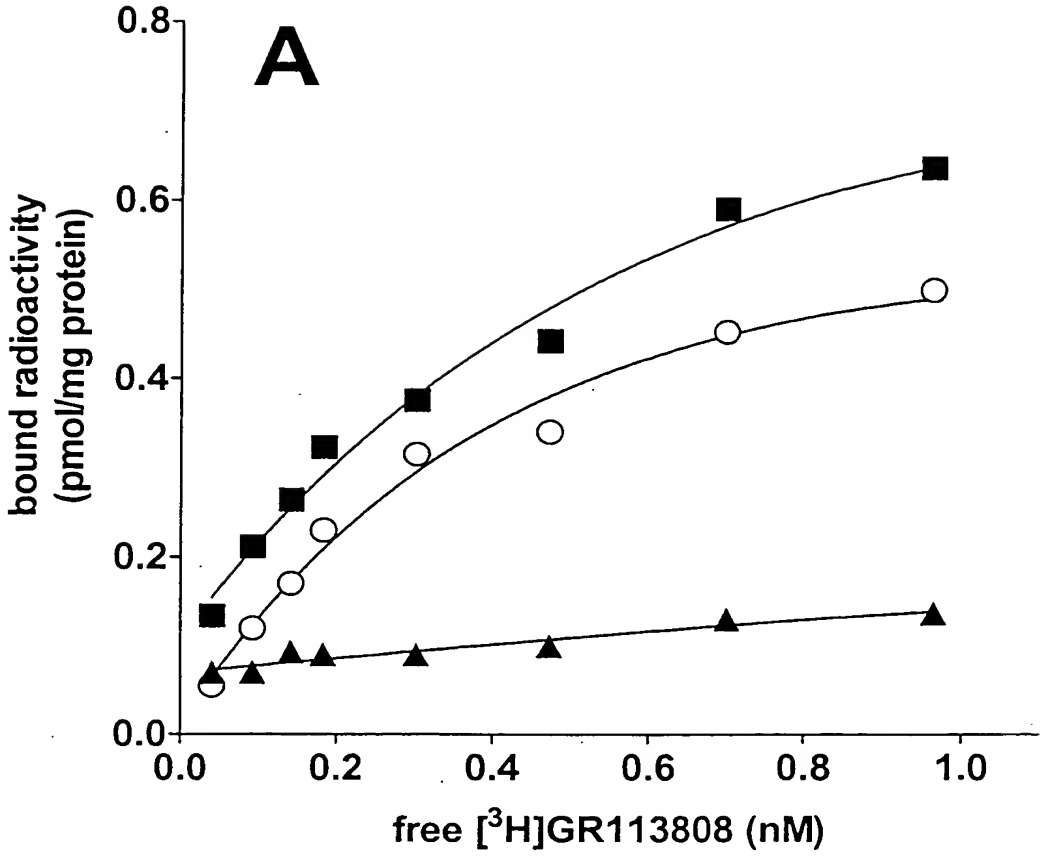

A
0.8
0.6
0.4
0.2
0.0
bound radioactivity
(pmol/mg protein)
0.0
0.2
0.4
0.6
0.8
1.0
free [3H]GR113808 (nM)

Fig 3a

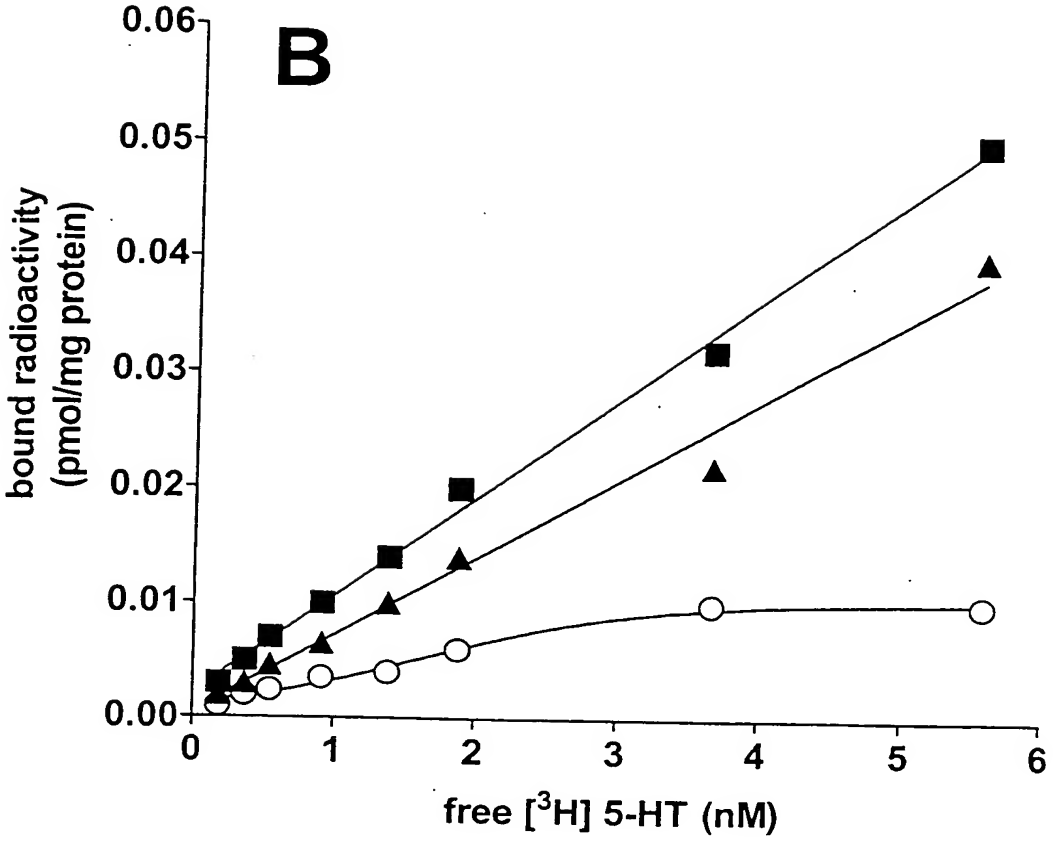
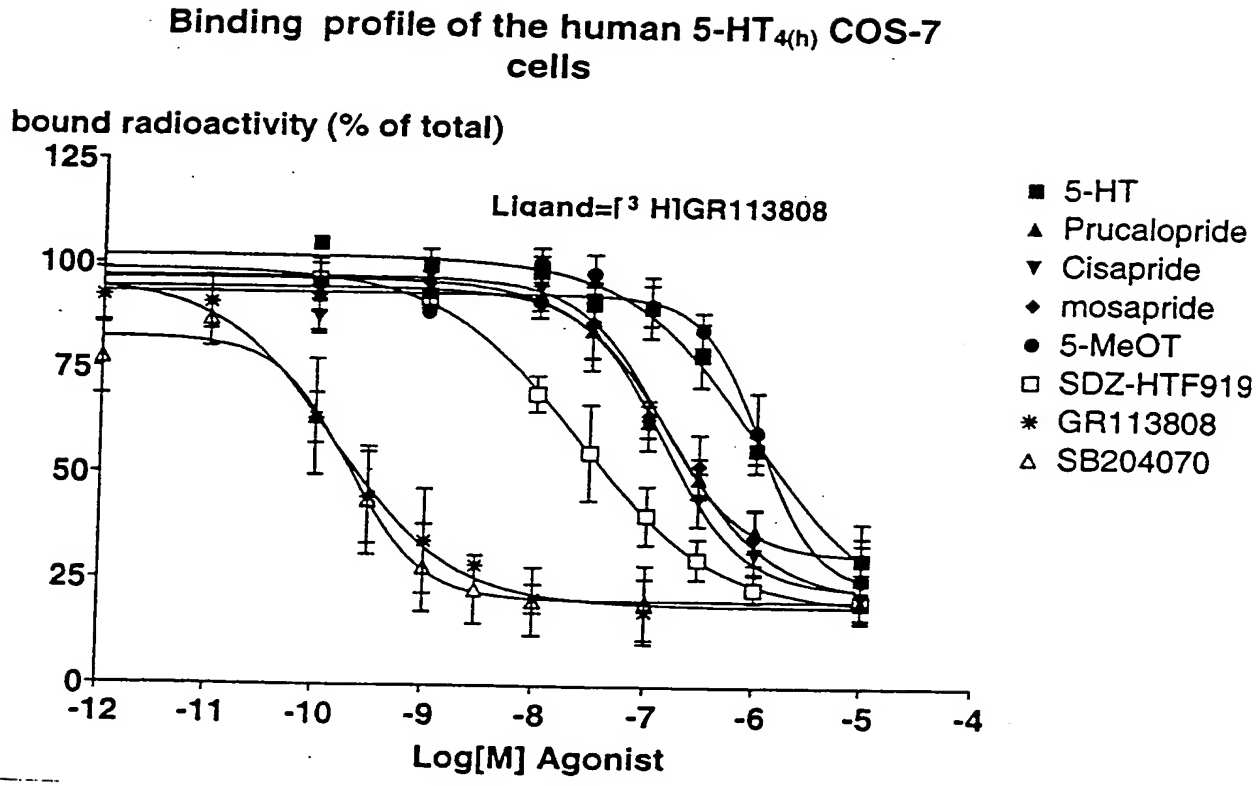

B
0.06
0.05
0.04
0.03
0.02
0.01
0.00
bound radioactivity
(pmol/mg protein)
0
1
2
3
4
5
6
free [3H] 5-HT (nM)

Fig 3b

Fig. 4.


Binding profile of the human 5-HT4(h) COS-7 cells
bound radioactivity (% of total)
Ligand=[3 H]GR113808
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
-4
Log[M] Agonist
5-HT
Prucalopride
Cisapride
mosapride
5-MeOT
SDZ-HTF919
GR113808
SB204070

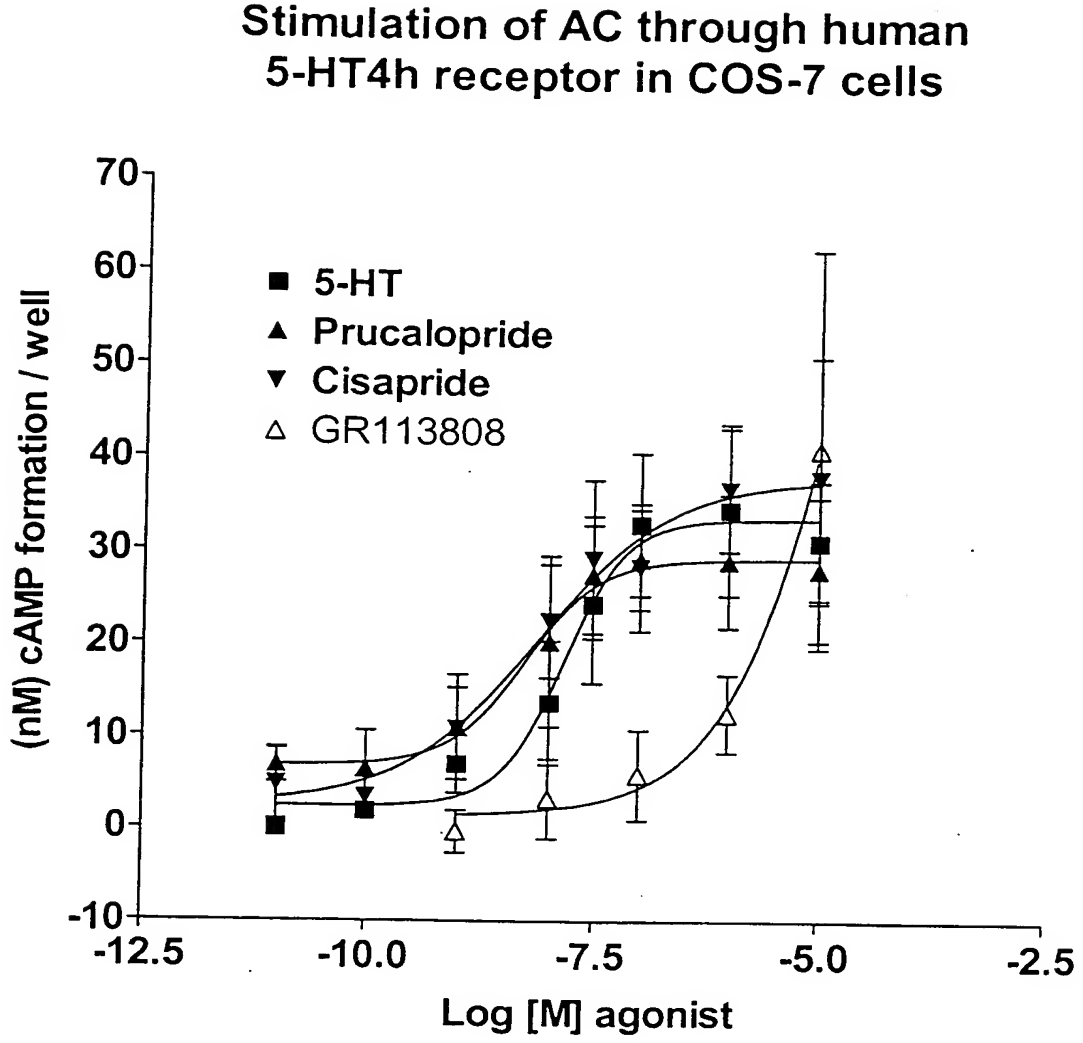

Stimulation of AC through human
5-HT4h receptor in COS-7 cells
(nM) cAMP formation / well
70
60
50
40
30
20
10
0
-10
-12.5
-10.0
-7.5
-5.0
-2.5
Log [M] agonist
5-HT
Prucalopride
Cisapride
GR113808

Fig 5